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Survival and Growth of *Salmonella* Enteritidis in Liquid Egg Products Varying by Temperature, Product Composition, and Carbon Dioxide Concentration

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Abstract

Cryogenic cooling of shell eggs with carbon dioxide (CO_2) is known to improve egg content quality through rapid cooling as well as by increasing internal CO_2 levels. A study was undertaken to determine the effects of variations in atmospheric CO_2 concentrations (aerobically stored, flushed with CO_2 and sealed, or bubbled with CO_2) on the survival and growth of *Salmonella* Enteritidis in liquid egg products including whole egg, albumen, yolk, and albumen + 1% yolk. Egg products were inoculated with a three-strain composite of *Salmonella* Enteritidis at ca. 4 log colony-forming units (CFU)/mL and stored at 7°C or 10°C for 8 or 4 days, respectively, or at ca. 2 log CFU/mL and stored at 23°C and 37°C for 48 or 24 hours, respectively. *Salmonella* populations differed based on variations in liquid egg composition ($p < 0.05$). Manipulating the atmospheric concentrations of CO_2 in which liquid egg products were stored did not significantly inhibit the growth of *Salmonella* Enteritidis ($p > 0.05$) in yolk-containing egg products or affect the inhibitory activity of albumen-containing products. Populations of *Salmonella* were static at 7°C over the entire storage period and significant growth occurred in whole egg and yolk stored at 10°C. Populations in egg stored at 23°C and 37°C were greater in yolk than in whole egg, although whole egg had populations greater than in albumen or albumen +1% yolk ($p < 0.05$). Results of this investigation suggest that increasing atmospheric CO_2 to enhance egg quality should not promote the growth of *Salmonella* Enteritidis in eggs.

Introduction

Foodborne illnesses that originate from eggs containing *Salmonella* Enteritidis continue to be of public concern. The U.S. Department of Agriculture (USDA), Food Safety and Inspection Service, based on current epidemiological surveillance data, calculates that 130,000 human *Salmonella* Enteritidis–related illnesses occur each year with annual associated costs of US$1.8–3.1 billion (FDA, 2004; Schroeder *et al.*, 2006). Between 1985 and 1998, 841 outbreaks of *Salmonella* Enteritidis were reported to the Centers for Disease Control and Prevention including 79 deaths, 2904 hospitalizations, and 29,762 illnesses (Patrick *et al.*, 2000). Eighty percent of these illnesses for which information was available were egg related (Patrick *et al.*, 2000). St. Louis *et al.* (1988) first reported that these illnesses, which peaked in the mid- to late 1980s, stemmed from clean, sanitized, whole, intact, and internally contaminated grade A shell eggs that had been processed according to federal regulations. *Salmonella* is known to infect the ovaries and other reproductive organs of the hen (Timoney *et al.*, 1989; Miyamoto *et al.*, 1996; Gast *et al.*, 2007). Studies have demonstrated that layer–breeder hens may lay *Salmonella* Enteritidis–infected eggs leading to vertical transmission of *Salmonella* from breeder hens to chicks destined to become layers (Humphrey *et al.*, 1991). It is estimated that 1 in every 20,000 table eggs produced in the United States, or 3.25 million eggs per year, is positive for *Salmonella* Enteritidis (Ebel and Schlosser, 2000); thus, processing strategies to control the growth of the pathogen in eggs are critical.

Factors that influence the motility and multiplication of *Salmonella* Enteritidis within eggs may include temperature, pH, and carbon dioxide (CO_2) concentrations. Heath (1977) noted that as the pH within the egg increases, the sulfhydryl content also increases, which may lead to liquefaction of albumen due to the uncoiling of proteins. After an egg has been

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laid, the pH of albumen will increase from ca. 7.6 to a peak of between 9.0 and 9.5 during long-term storage (Goodrum *et al.*, 1989; Stern, 1991). Maintaining a low pH within the avian egg is essential to preserving high egg quality aspects such as vitelline membrane strength, Haugh unit measurements, and USDA egg grades for quality defects, which improve with increasing concentrations of CO_2. Within 7 days of lay, in the presence of CO_2, albumen quality declines little if any (Walsh *et al.*, 1995). Brake *et al.* (1993) suggested that higher albumen quality also delays gaseous exchange, while Walsh (1992) explained that shell egg gas influx (i.e., loss of CO_2 and increase in oxygen) enhances dissolution of the albumen gel (cf. Vick *et al.*, 1993). This liquefaction may be a primary cause of *Salmonella* Enteritidis migration from the albumen to the nutrient- and iron-rich yolk leading to logarithmic growth (Hammack *et al.*, 1993; Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995). One study observed that liquefaction of egg albumen did not increase commensurate with storage temperature when pH levels remained close to original values of ca. 7.6 (Cotterill *et al.*, 1959).

The temperature at which eggs are stored affects growth of *Salmonella* Enteritidis, which has been shown to grow more rapidly as the temperature increases from 10°C to 25°C (Gast and Holt, 2000). Foodborne outbreaks seem to be seasonal, peaking in warmer summer months, which could be a function of reduced egg cooling efficiency during these periods (Kim *et al.*, 1989). Saeed and Koons (1993) inoculated 20 colony-forming units (CFU)/egg of *Salmonella* Enteritidis into the yolk of shell eggs and stored them at 23°C and 4°C. Populations of *Salmonella* Enteritidis in eggs held for 24 hours at 23°C increased to 7 log CFU/mL of egg and after 72 hours reached 9 log CFU/mL of egg. Eggs stored at 4°C, however, promoted very little growth of *Salmonella* Enteritidis up to 15 days of storage.

Curtis *et al.* (1995) demonstrated that rapid CO_2 cryogenic cooling has a profound effect on reducing bacterial growth and increasing egg quality. Internal and external bacterial counts of *Pseudomonas fluorescens* (ATCC no. 17400) were lower in CO_2 cryogenically cooled eggs than in traditionally cooled eggs. Keener *et al.* (2000a, 2000b) quantified the relationship between cryogenic versus traditional egg cooling based on CO_2 concentrations within the egg air cell. Eggs that were heated prior to cryogenic cooling, as occurs during shell sanitization, had lower CO_2 levels (0.04%) than eggs not heated before cooling (0.08%) (Keener *et al.*, 2000b). The CO_2 concentrations of eggs that were cooled with cryogenic CO_2 and stored in a CO_2-rich atmosphere increased from 0.04% to 48% along with an increase in egg Haugh values (Keener *et al.*, 2000b). Walsh *et al.* (1995) reported that eggs stored in the presence of CO_2 experience an increase in quality as determined by albumen height after 7 and 14 days of storage. Jones *et al.* (2002) reported that Haugh units were greater for CO_2 cryogenically cooled eggs than for traditionally cooled eggs. It is clear that increasing CO_2 within the egg leads to an improvement in quality, although little is known about the survival of *Salmonella* Enteritidis in egg components as affected by increased CO_2 concentrations. We undertook a preliminary study with the objective of determining how variations in CO_2 concentrations would affect the survival and growth of *Salmonella* Enteritidis in whole egg, yolk, albumen, and a matrix representative of egg white contaminated with yolk (albumen + 1% yolk).

Materials and Methods

Egg preparation

Eggs were collected from a flock of *Salmonella* Enteritidis–free Hy-Line (West Des Moines, IA) single-combed white Leghorn laying hens within 18 hours of lay, and were allowed to equilibrate to room temperature (22°C). Eggs were examined by visual surface evaluation as well as internally by candling, and all cracked, checked, and loss eggs were removed. Eggs were then individually immersed in a 10% household bleach solution (equivalent to 0.525% sodium hypochlorite) adjusted to 30°C with sterile forceps followed by immersion in a 70% ethanol solution, flaming over an open bunsen burner, and resting on sterile aluminum foil. Eggs were aseptically opened with sterile scalpels and tweezers and contents were either emptied as whole egg, or aseptically separated into representative yolk and albumen fractions with a sterile egg yolk separator.

Egg contents were divided into four groups: whole egg, yolk, albumen, and albumen +1% yolk.

Each egg fraction was placed in sterile glass blending jars, respectively, and agitated with a Waring blender (Waring, New Hartford, CT) set at the lowest speed for 2 minutes. Care was taken to prevent shearing of albumen proteins. Blended egg samples (100 mL) were placed into sterile 250-ml flasks.

Preparation of inocula

A composite suspension of nalidixic acid–resistant *Salmonella* Enteritidis strains Benson, Puerto Rico, and Rochester (provided by Nelson A. Cox, USDA Agricultural Research Service, Athens, GA) were grown in brain heart infusion broth at 37°C with loop transfers (ca. 10 μL) at 24-hour intervals to produce a final concentration of ca. 9 log CFU/mL from which inocula were prepared. Cells were washed with sterile deionized water by centrifugation three times in an IEC Centra MP4 centrifuge (International Equipment Company, Needham Heights, MA).

Inoculation protocol

All 150-mL egg samples were inoculated in flasks with 1 mL of inoculum at 22°C and mixed by agitation for 1 minute prior to storage. Yolk samples were mixed by agitation as well as with a sterile 10-mL pipette. Inoculum levels were confirmed by spiral plating the suspension onto brilliant green sulfa agar (BGS, Difco, Becton Dickinson, Sparks, MD) containing 200 μg/mL nalidixic acid (BGSN) with a Whitley automatic spiral plater (Spiral System Instruments, DW Scientific Ltd., West Yorkshire, England) on the 50-μL setting, incubating at 37°C for 24 hours, and enumerating with a Spiral Systems Model 500A bacterial colony counter (Exotech, Inc. Gaithersburg, MD).

Carbon dioxide treatments and storage

Liquid egg products stored at 7°C and 10°C were inoculated with *Salmonella* Enteritidis suspensions at populations of ca. 4 log CFU/mL, and egg products stored at 23°C and 37°C were inoculated at populations of ca. 2 log CFU/mL. Inoculum levels were chosen to allow measurement of fluctuations in *Salmonella* populations stored over time. Samples inoculated at 4 log CFU/mL were stored at 7°C for 0, 2, 4, 6,

and 8 days, or at 10°C for 0, 1, 2, 3, and 4 days. Samples inoculated at 2 log CFU/mL were stored at 23°C for 0, 12, 24, 36, and 48 hours, or at 37°C for 0, 4, 6, 8, 10, 12, and 24 hours. Temperatures were chosen to simulate mandated refrigeration temperature for eggs (7°C), a temperature-abused condition (10°C), a common room temperature (23°C), and the optimal temperature for the growth of *Salmonella* (37°C).

Liquid egg products were subjected to one of three atmospheric treatments: aerobically stored, flushed with CO_2 for 10 seconds and hermetically sealed, or continuously bubbled with CO_2. Aerobic treatments were sealed with a sterile, breathable foam stopper. The second set of flasks were flushed with CO_2 (BOC Gases, Murray Hill, NJ), that had been filtered through a 3-μm filter and sealed with a sterile rubber stopper. Continuously bubbled CO_2 samples were treated by inserting two lengths of glass tubing into a rubber stopper to allow continuous gas exchange. The longer piece of glass tubing protruded 2.5 cm above the top of the rubber stopper and descended down to the bottom of the flask when capped. Rubber tubing was affixed to a CO_2 tank regulator, connected to the glass tubing that protruded from the stopper, and CO_2 was added to liquid egg fractions at a rate of about one bubble per second. A second shorter piece of glass tubing, also affixed through the rubber stopper, protruded ca. 1 cm above and below the stopper and was filled with sterile cotton to provide a continuous flow of CO_2 through the system.

Microbiological analysis

Duplicate samples were obtained from each flask and analyzed for populations of *Salmonella* Enteritidis at each sampling time. Day 0 and hour 0 samples were analyzed within 1 hour of inoculation. Samples held at 7°C were tested at 2-day intervals for 8 days, 10°C treatments were sampled daily up to day 4, 23°C treatments were sampled every 12 hours up to 48 hours, and 37°C treatments were sampled at 0, 4, 6, 8, 10, 12, and 24 hours. Each flask was agitated and 1 mL was aseptically removed with a pipette while continuing to agitate the flask. Samples were serial diluted into Butterfield's diluent blanks (9 mL) and spiral plated (50 μL) onto BGSN. One milliliter of a 1:9 (egg/Butterfield's diluent) dilution of each liquid egg sample was also pour plated with ca. 10 mL of molten BGSN (45°C) and allowed to solidify. All plates were incubated at 37°C for 24 hours and typical *Salmonella* Enteritidis colonies were enumerated.

Statistical analysis

Experiments were performed in triplicate. Data were expressed as $\log_{10}$ CFU/mL and analyzed with a Completely Randomized Design with repeated measure and nested treatment arrangements; time intervals nested in storage temperatures. Statistical analysis was performed with the SAS Mixed Procedures using SAS Software Release 8.0 (Statistical Analysis System, SAS Institute, Cary, NC) Significant difference among means were determined the Bonferroni LSD technique. Significance was set at $p \leq 0.05$.

Results and Discussion

Effect of CO_2 treatment

Eggs stored in the presence of CO_2 are known to increase in quality as determined by albumen height (Walsh *et al.*, 1995). Manipulating the atmospheric concentrations of CO_2 in which liquid egg products were stored in the present study did not significantly inhibit the growth of *Salmonella* Enteritidis ($p > 0.05$) in yolk-containing media or affect the inhibitory activity of albumen-containing egg products over time or at any sampling time throughout the storage period (Table 1).

TABLE 1. MEAN POPULATION OF *SALMONELLA* ENTERITIDIS INOCULATED INTO LIQUID EGG PRODUCTS AND STORED AT 7°C, 10°C, 23°C, AND 37°C FOR 8 DAYS, 4 DAYS, 48 HOURS, AND 24 HOURS, RESPECTIVELY, UNDER VARIOUS CONCENTRATIONS OF CARBON DIOXIDE

		Population (log CFU/mL) recovered[a]		
Egg product	*Storage temperature (°C)*	*Aerobic*	*Flushed with CO_2 and sealed*	*Bubbled with CO_2*
Whole egg	7	3.91 a	4.00 a	3.83 a
	10	4.32 a	4.31 a	4.31 a
	23	3.94 a	4.05 a	3.95 a
	37	3.38 a	3.30 a	3.47 a
Yolk	7	3.87 a	3.79 a	3.87 a
	10	4.53 a	4.56 a	4.65 a
	23	5.57 a	5.58 a	5.47 a
	37	4.81 a	4.65 a	4.82 a
Albumen	7	3.25 a	3.41 a	3.30 a
	10	3.54 a	3.66 a	3.61 a
	23	1.73 a	1.71 a	1.85 a
	37	3.17 a	3.19 a	3.17 a
Albumen + 1% yolk	7	3.47 a	3.41 a	3.45 a
	10	3.84 a	3.75 a	3.73 a
	23	2.21 a	2.06 a	2.13 a
	37	3.15 a	3.17 a	3.07 a

[a]Mean values in the same row that are not followed by the same letter are significantly different ($p \leq 0.05$). Mean values represent averages from duplicate samples in three replicate experiments over time.

These data are in contrast to other results obtained with shell eggs cooled with CO_2 (Hughes, 1999), although results from the present study may not reflect the situation in intact shell eggs based on differences in methodology. Loss of CO_2 in shell eggs has been correlated with oxygen influx, which increases at lower wet bulb (lower relative humidity) readings in hatching eggs (Vick *et al.*, 1993). This exchange of gases is known to be greatest at the beginning and end of the hen's egg laying cycle when eggshell porosity is highest (Peebles and Brake, 1985, 1987). The ability of eggs to support gaseous exchange during cryogenic CO_2 cooling and affect *Salmonella* growth, therefore, may be a function of the age of the layer flock. *Salmonella, as* a genus within the bacterial family Enterobacteriaceae, is facultatively anaerobic. The ability of *Salmonella* Enteritidis to persist in egg products at elevated CO_2 concentrations, to our knowledge, has not heretofore been reported. Results in the present study indicate that the survival and growth of *Salmonella* Enteritidis is not affected by fluctuations in CO_2, indicating that manipulating the concentrations of CO_2 in order to enhance the quality of egg products should not compromise the food safety of eggs with respect to *Salmonella* Enteritidis. These results should not necessarily be interpreted as being representative of the elevated-CO_2 response of the entire genus *Salmonella*, which is known to have more than 2500 serotypes (Popoff, 2001; Popoff *et al.*, 2003; Humphrey, 2004).

Recovery of cells from liquid egg products stored at 7°C and 10°C

Differences in population of *Salmonella* Enteritidis were apparent based on variation in liquid egg composition at all temperatures (Figs. 1–4). Populations of *Salmonella* Enteritidis in whole egg and yolk held at 7°C were slightly greater than populations in albumen and albumen +1% yolk at 1, 2, 4, and 8 days of storage (Fig. 1); however, populations of all egg products generally remained static over the storage period. Murase *et al.* (2005) reported slight growth of *Salmonella* Enteritidis in 5% of inoculated albumen samples held at 4°C for up to 4 weeks.

Our results from samples held at 10°C indicate that *Salmonella* Enteritidis populations in albumen and albumen +1% yolk did not increase up to day 4 (Fig. 2). Populations in yolk increased from 4.0 to 5.2 log CFU/mL during this same time period, which was significantly greater than the increase of *Salmonella* Enteritidis counts (4.2 to 4.6 log CFU/mL) in whole egg. Our findings are similar to those of Gast and Holt (2000) who reported up to 1 log CFU/mL increases in *Salmonella* Enteritidis counts in yolk between days 1 and 3 of storage at 10°C.

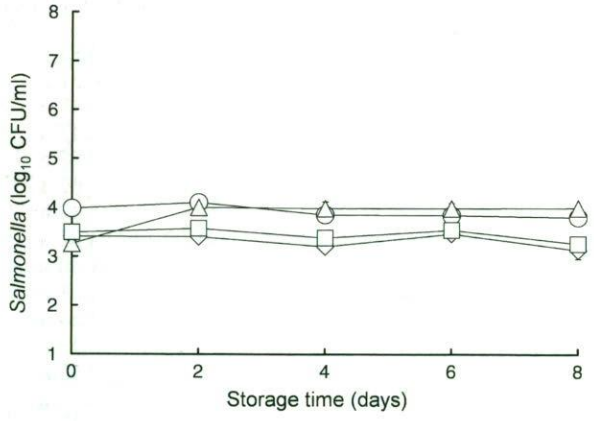

FIG. 1. Populations of a three-strain composite of *Salmonella* inoculated (ca. 4 log CFU/mL) into liquid whole egg (○), albumen (◇), yolk (△), or albumen +1% yolk (□), and stored at 7°C for up to 8 days.

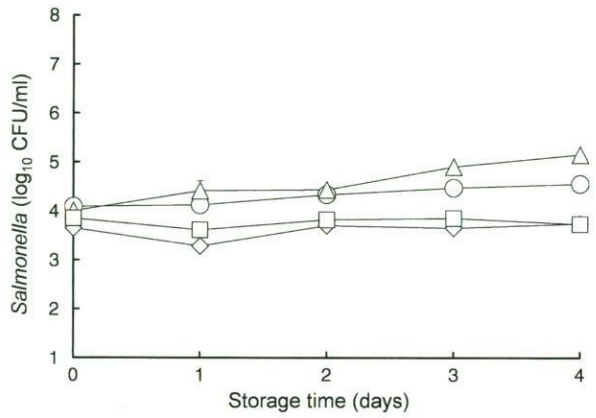

FIG. 2. Populations of a three-strain composite of *Salmonella* inoculated (ca. 4 log CFU/mL) into liquid whole egg (○), albumen (◇), yolk (△), or albumen +1% yolk (□), and stored at 10°C for up to 4 days.

Recovery of Salmonella *Enteritidis from liquid egg products stored at 23°C*

Although inoculum levels were identical for all liquid egg products within this category, fewer cells were recovered at hour 0 from albumen and albumen +1% yolk than from yolk or whole egg (Fig. 3). This initial reduction may be due to the inactivation properties of antimicrobial compounds in albumen (Stephenson *et al.*, 1991; Connor, 1993; Baron *et al.*, 1997; Froning, 2002). Published results of the survival and growth of *Salmonella* in albumen have been mixed. Gast and Holt (2001) reported that counts of 12 strains of *Salmonella*

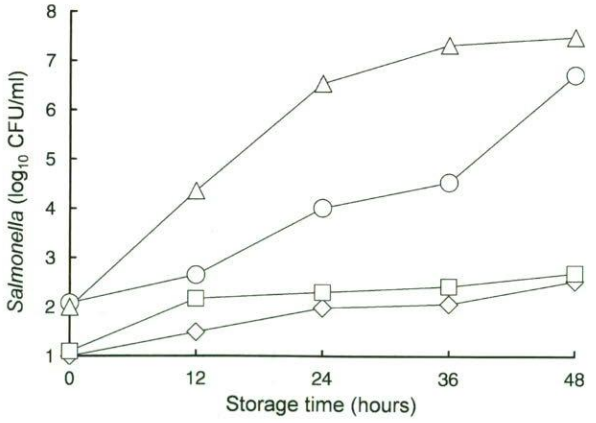

FIG. 3. Populations of a three-strain composite of *Salmonella* inoculated (ca. 2 log CFU/mL) into liquid whole egg (○), albumen (◇), yolk (△), or albumen +1% yolk (□), and stored at 23°C for up to 48 hours. The maximum detection limit was 7.48 log CFU/mL.

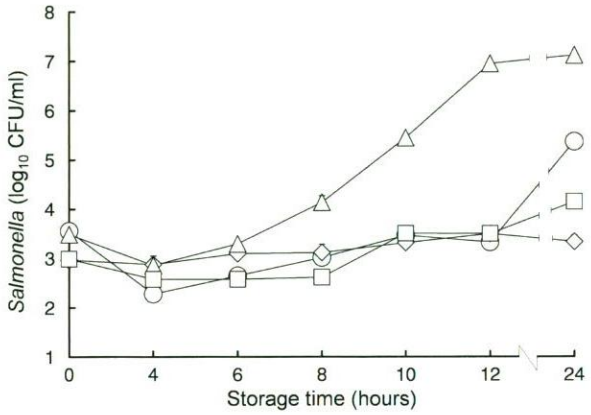

FIG. 4. Populations of a three-strain composite of *Salmonella* inoculated (ca. 2 log CFU/mL) into liquid whole egg (○), albumen (◇), yolk (△), or albumen +1% yolk (□), and stored at 37°C for up to 24 hours. The maximum detection limit was 7.48 log CFU/mL.

Enteritidis inoculated into albumen at a population of 5 log CFU/mL and stored at 25°C were reduced by up to 1.2 log CFU/mL after 24 hours of storage. Humphrey and Whitehead (1993) reported that little growth of *Salmonella* Enteritidis occurred when the bacterium was inoculated into albumen and stored at 20°C; yet, populations increased more rapidly when eggs were stored for at least 21 days prior to inoculating the albumen. Another study (Messens *et al.*, 2004) reported that the growth of *Salmonella* in albumen at 20°C was higher in fresh albumen than in albumen held for up to 3 weeks before inoculation. These results were partially attributed to a rise in pH from 8.16 in fresh eggs to 9.26 for eggs stored for 2 weeks prior to inoculation. Lock and Board (1992) reported that populations did not increase for a majority of *Salmonella* serotypes inoculated into albumen and held at 4°C, 20°C, or 30°C for 42 days.

In the current study, we found that numbers of *Salmonella* Enteritidis in albumen and albumen +1% yolk increased by ca. 1–2 log CFU/mL (close to the inoculum level) in 24 hours and increased by another ca. 0.5 log CFU/mL within 48 hours of storage. Chen *et al.* (2005) indicated that *Salmonella* Enteritidis inoculated into the albumen of shell eggs held at 22°C increased by ca. 1.5 and 5 log CFU/mL after 1 and 3 weeks of storage, respectively (Chen *et al.*, 2005). Other studies found that *Salmonella* Enteritidis populations inoculated into albumen and held at 30°C were able to increase by up to 1 log and 3 log CFU/mL within 24 and 96 hours of storage, respectively (Baron *et al.*, 1997, 2004). Růzicková (1994) reported that populations of *Salmonella* Enteritidis strain 2553 in whole egg increased by up to 6 log when stored at 21°C for 24 hours. Baron *et al.* (1999) found that *Salmonella* Enteritidis inoculated into laboratory-collected egg white and egg white collected at a processing factory increased by 1 and 2.5 log CFU/mL, respectively, when stored at 30°C for 24 hours. The authors concluded that differences in growth may be attributed to the unintentional increase in iron concentrations during factory egg breaking, which could saturate and inhibit iron-binding egg proteins. The addition of 1% iron-rich yolk to albumen in the present investigation, however, did not enhance the growth of *Salmonella* Enteritidis in a similar fashion and discrepant results may be due to differences in bacterial strains and storage temperatures. Nevertheless, our findings were similar to those of Messens *et al.* (2004) who found that storing the albumen in the presence or absence of yolk did not affect the growth of *Salmonella*.

In the present study, populations of *Salmonella* stored at 23°C (Fig. 3) were significantly different between yolk and whole egg, and between whole egg and albumen +1% yolk at 12, 24, 36, and 48 hours, although populations in albumen +1% yolk and albumen did not differ statistically. Populations in yolk and whole egg increased by up to 5.5 and 4.7 log CFU/mL, respectively, during 48 hours of storage. *Salmonella* Enteritidis populations may increase with increasing concentrations of iron-rich yolk as a result of the saturation of chelating proteins (e.g., ovotransferrin) by iron in addition to yolk providing a rich source of growth-promoting nutrients.

Recovery of Salmonella *Enteritidis from liquid egg products stored at 37°C*

Salmonella populations in albumen +1% yolk increased by 1.57 log CFU/mL between 4 and 24 hours of storage while numbers in albumen remained virtually unchanged during the same period of time (Fig. 4). Populations in whole egg stored at 37°C increased to 3.47 log CFU/mL by 10 hours of storage with a generation time of 90.9 minutes between 4 and 10 hours, and by 24 hours, populations in whole egg reached 5.37 log CFU/mL. Gast and Holt (1995) reported that 12 strains of *Salmonella* Enteritidis individually inoculated into separate samples of whole egg at a population of 5 CFU/mL and stored at 37°C increased to levels of between 3.9 and 7.5 log CFU/mL within 24 hours, demonstrating the disparity in growth characteristics between strains of *Salmonella*. In the current investigation, we found that growth of *Salmonella* Enteritidis stored at 37°C was more robust in yolk than in whole egg, increasing to a population of 6.95 log CFU/mL with a generation time of 34.9 minutes between 4 and 12 hours of storage. Růzicková (1994) reported that *Salmonella* Enteritidis populations in yolk exhibited an up to 7 log increase when stored at 37°C for 24 hours, and ca. 1.25 log increase in albumen stored for 24 hours at 21°C; however, *Salmonella* Enteritidis growth was inhibited in albumen held at 8°C or 37°C for 24 hours.

The results of the present study suggest that manipulating atmospheric CO_2 levels at which eggs are stored to enhance egg quality (Walsh *et al.*, 1995; Keener *et al.*, 2000a, 2000b; Jones *et al.*, 2002) should not promote the growth of *Salmonella* Enteritidis in eggs contaminated at levels as high as 2–4 log CFU/mL. More research, however, is warranted to determine the effects and interactions of CO_2 concentrations on various *Salmonella* Enteritidis strains and *Salmonella* serovars, which are known to vary in individual growth patterns when inoculated into liquid egg products (Gast and Holt, 1995; Murase *et al.*, 2006).

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names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Disclosure Statement

No competing financial interests exist.

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